Urogenital Paraneurons in Several Mammals*

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Summary. Amines and/or peptide-producing cells, deserving to be called paraneurons, were demonstrated in the urethro-prostatic complex of the man, rabbit, buffalo and sheep and in the uterine horns of the pig, horse and mouse, by means of histochemical, immunohistochemical, immunofluorescent and double labeling immunofluorescent techniques. In particular, the urethro-prostatic complex of the sheep contains cells producing serotonin, chromogranin A and somatostatin. Often the amine and the "marker"-protein were colocalized in the same cells. Chromogranin A- and somatostatin-containing cells were found in the uterine horns of the pig.

Serotonin probably plays a role in the contraction/relaxation of the musculature of the male urogenital tract, thus regulating the urinary and spermatic flow.

The function of somatostatin cells in the urogenital tract is unknown. The presence and direction of their cytoplasmic processes lead us to hypothesize a double endocrine/paracrine modality of action.

The discovery of serotonin within enterochromaffin cells (VIALLI and ERSPAMER, 1939; ERSPAMER and ASERO, 1952), the argyrophilia of several urogenital "helle Zellen" supposed to produce hormones (FEYRTER, 1951), the description of central neurons capable of secretion (E. SCHARRER, 1928), the recognition of peptidergic neurons (BARGMANN et al., 1967) and the subsequent conceptual fusion of both nervous and endocrine systems in a unique morphofunctional complex termed "neuroendocrine" by E. and B. SCHARRER (1963) and then "diffuse neuroendocrine system" (DNES) by PEARSE (1977), have all induced progressive modifications of the classical concepts regarding the physiology of such recepto-secretory cells, as well as their terminology. On the basis of an integrated phylogenetic and morpho-functional study, such cells have been called "paraneurons" by FUJITA (1976, 1980). Paraneurons are diffused throughout the

body and reach their target organs and/or cells by means of physiologically active substances used as signals for their communications. Such substances are generally aminic, nucleotidic or peptidic in nature.

In the male urogenital tract, paraneurons were firstly described by FEYRTER (1951) as endocrine-like "helle Zellen" isolated among the epithelial cells of the human urethra and prostate. Urogenital paraneurons have been successively described in the urethro-prostatic complex of many animal species by means of histochemical techniques (OWMAN et al., 1971; DIXON et al., 1973; HÅKANSON et al., 1974; VITTORIA et al., 1983; PAINO et al., 1985). More recently, immunohistochemistry has allowed us to demonstrate within human urogenital paraneurons under normal conditions, the following bioactive substances: 5-hydroxytryptamine (5-HT) or serotonin (FETISSOF et al., 1983; DI SANT'AGNESE et al., 1985; ABRAHAMSSON et al., 1986; IWANAGA et al., 1987), somatostatin (DI SANT'AGNESE and DE MESY JENSEN, 1984), calcitonin (FETISSOF et al., 1986a; DI SANT'AGNESE, 1986), bombesin (DI SANT'AGNESE, 1986), α -HCG (FETISSOF et al., 1987) and TSH (ABRAHAMSSON et al., 1986). In non-human species serotonin has been immunohistochemically described in the male urethro-prostatic complex of the sheep (VITTORIA et al., 1985), buffalo (VITTORIA et al., 1986a) and dog (HANYU et al., 1987). Morphological studies performed in the female urogenital tract are less numerous and often describe pathological conditions such as a carcinomatous uterus. However, by means of immunohistochemical techniques, serotonin has been described in paraneurons of the normal cervix and vagina in woman (FETISSOF et al., 1985) and of the urethra and vestibulum in the dog (HANYU et al., 1987).

Since the early 1980s we have been trying to reduce the gap between the findings on human and non-

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human neuroendocrine cells in urogenital organs, focusing in particular on possible evolutionary and/or species-dependent differences. On this basis, the presence and morphological peculiarities of some paraneurons in the urogenital tract of eight mammalian species are described here by means of histochemical, immunohistochemical and immunofluorescent techniques.

MATERIAL AND METHODS

Samples were taken from the pelvic urethra and prostate of the rabbit, sheep and buffalo. From the sheep and buffalo samples of bulbo-urethral glands were taken as well. Surgical specimens were collected from hyperplastic, non-carcinogenic prostates of humans, and only tissues devoid of pathological alterations were utilized.

For the female urogenital tract, fragments were collected from the uterine horns and body of non-pregnant and non-castrated pigs, horses and mice. Samples from the uterus of a pseudo-hermaphroditic pig were taken as well. This specimen was 127 days old and possessed a well developed uterus annexed to a normal male genital apparatus.

The material was fixed in Bouin's fluid, paraformaldehyde 4% in 0.1 M phosphate buffer, pH 7.3, glutaraldehyde 6% in 0.1 M phosphate buffer, pH 7.4, or formaldehyde 10%, soon after collecting. It was routinely dehydrated, embedded in Paraplast and cut at 4 μ m.

Sections were stained by the lead-haematoxylin method (SOLCIA *et al.*, 1969), by the argentaffin tech-

nique of Masson-Hamperl, modified by SINGH (1964), and by the argyrophil methods following GRIMELIUS (1968), Bodian modified by SINGH (1962) and LINDER (1978). The latter has been only recently introduced to the study of neuroendocrine cells (CECIO *et al.*, 1985, 1988a).

Other sections were stained by the peroxidase-antiperoxidase (PAP) method of STERNBERGER (1979) or by the indirect immunofluorescent technique of COONS (1956). The primary antibodies used and the relative dilutions and times of incubation are reported in Table 1. The site of the antigen-antibody reaction was revealed by diaminobenzidine (DAB) or aminoethylcarbazole (AEC) for the PAP method and by secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or with lissamine-rhodamine isothiocyanate (LRITC) for the indirect immunofluorescence. Control sections were obtained by preabsorbing each antibody with the relative antigen and applying such a complex on sections in the first immunological step instead of the antibody alone. Control sections consistently gave negative results.

The colocalization of 5-HT and chromogranin A in the same cells has been studied by a double labeling immunofluorescent technique (WESSENDORF and ELDE, 1985), where the two primary antibodies are raised in different animal species and then applied simultaneously to a section. The secondary antibodies were also used simultaneously and conjugated with the fluorochromes described above. Sections were observed by a Leitz microscope equipped with a reflecting system for fluorescence using two filter sets, the first for FITC (450-490 nm bandpass excitation-, and 525/20 nm bandpass barrier-filter) and the

Table 1. Specifics of the antisera used

Antigen	Raised in	Code no.	Dilution	Incubation time	T °C	Source
Chromogranin A	Rabbit	20086	1 : 100-1 : 500	1.5 h	37	Immunonuclear Corp. (Stillwater, MN)
5-HT	Rabbit	20080	1 : 100-1 : 500	1.5 h	37	Immunonuclear Corp.
5-HT	Goat	20079	1 : 100	1.5 h	37	Immunonuclear Corp.
Somatostatin 1-14	Rabbit	A 556	1 : 300	16 h	4	Dako/Accurate (Westbury, NY)
Bombesin	Rabbit	30H2T	1 : 100	24 h	4	Immunonuclear Corp.
ACTH	Rabbit	24H2T	1 : 100	24 h	4	Immunonuclear Corp.
Calcitonin	Rabbit	25H2T	1 : 100	24-48 h	4	Immunonuclear Corp.
Gastrin 1-17	Rabbit	05H2T	1 : 100	24 h	4	Immunonuclear Corp.
Substance P	Rabbit	20064	1 : 100	24 h	4	Immunonuclear Corp.
VIP	Rabbit	20077	1 : 100	24 h	4	Immunonuclear Corp.

second for LRITC (546/19 nm bandpass excitation, and 580 nm longpass barrier-filter). Using such filter sets eliminates any red emission during blue excitation and, conversely, green-yellow emission during green excitation. Controls were carried out as recommended by WESSENDORF and ELDE (1985), able to definitively clarify possible cross-reactions among primary and/or secondary antibodies. Such artifacts can simulate colocalization of 5-HT and chromogranin A.

The double immunofluorescent and the anti-5-HT-PAP-DAB stainings were performed on tissues from material fixed in 4% paraformaldehyde. The double technique was carried out on serial sections in order to correctly study colocalization of the substances in the same cells. Before such staining, several sections were incubated at 37°C with 0.06% trypsin in TRIS buffer (pH 7.6, 0.05 M) for 20 min.

Finally, sections from material fixed in 10% formalin were dewaxed, mounted and observed by a fluorescence microscope using a filter set effective for the study of biogenic amines.

RESULTS

The results concerning the male urogenital tract are

summarized in Table 2. The human prostate contains many paraneurons globous or irregular in shape. They often show dendrite-like processes extending between the basal membrane and the exocrine epithelium. In the peripheral zones of the gland, paraneurons are few, appearing isolated or in small clusters. Their number increases in the periurethral zone. Histochemical techniques showed that argyrophil paraneurons (Fig. 1a) are more numerous than argentaffin ones. The latter are also positive to the lead haematoxylin, probably because of their serotonin content.

The paraneurons found in the rabbit prostate (Fig. 1b) show distributions and shapes similar to those just described in humans but they are comparatively much less numerous. Urethral paraneurons (Fig. 1c) also appeared rather infrequently and often showed a roundish shape in the absence of evident cytoplasmic processes. As already described in the human, argyrophil paraneurons (Fig. 2a, b) numbered more than argentaffin ones (Fig. 2c, d).

Both the prostate (Fig. 1d) and pelvic urethra of the buffalo contain paraneurons regularly interposed between the basal membrane and the exocrine epithelium. They are particularly numerous in the primary and secondary excretory ducts of the gland and in the urothelium, but they appear to be comparatively less

Table 2. Paraneurons in the male genital tract

Species	Organ	Argyrophil stains			Argentaffin stain	Antibodies				
		Grimelius	Linder	Bodian		5-HT	Chrom. A	5-HT + Chrom. A	Sub. P; VIP	Somat. Bomb.; ACTH Gastr.; Calc.
Man	Prostate	++	++	+	++	?	?	?	?	?
Rabbit	Prostate	++	++	?	+	?	?	?	?	?
	Pelvic urethra	++	++	?	++	?	?	?	?	?
Buffalo	Prostate	++	++	?	+	+	?	?	?	?
	Pelvic urethra	++	++	?	++	++	?	?	?	?
	Bulbo-urethral glands	++	++	?	?	+	?	?	?	?
Sheep	Prostate	++	+++	+	++	++	+++	++	+	—
	Pelvic urethra	prox. 3rd	+++	+++	++	+++	+++	+++	+	—
		mid. 3rd	+++	+++	+	++	+++	++	+	—
		dist. 3rd	+	++	+	+	++	+	+	—

5-HT:serotonin, Chrom.: Chromogranin A, Somat.: somatostatin, VIP: vasoactive intestinal polypeptide, Sub. P: substance P, Bomb.: bombesin, ACTH:adrenocorticotrope hormone, Gastr.: gastrin, Calc.: calcitonin

?: not tested, —: not positive cells, +: rare positive cells, ++: few positive cells, +++: numerous positive cells, ++++: very numerous positive cells.

abundant than in the sheep. By means of immunohistochemistry, it was found that many of them contain serotonin generally diffused throughout the cytoplasm. Often such cells were multipolar in shape, with one of the cytoplasmic processes appearing constantly directed towards the lumen of the urethra or of the adenomeres. The excretory ducts of the buffalo bulbo-urethral glands contain numerous argyrophil and serotonin-immunopositive cells.

The urethro-prostatic complex of the sheep contains a greater number of paraneurons than that observed in the other species. For this reason we preferred the sheep for performing an accurate histochemical and immunohistochemical screening of such urogenital cells.

For sampling and description uniformity of the results obtained, we divided the pelvic urethra of the sheep craniocaudally into thirds: proximal, middle and caudal, respectively. It may be worthwhile to

recall that the proximal third contains the seminal collicle and that the prostate is diffused along the pelvic urethra from its precollicular zone up to the bulbo-urethral glands.

All histochemical techniques employed evidenced the occurrence of paraneurons in the urethroprostatic complex of the sheep (Fig. 2a, b). Immunohistochemically, neuroendocrine cells containing the amine 5-HT and the proteins chromogranin A and somatostatin were found in the said complex, while the antisera against substance P, VIP, bombesin, ACTH, gastrin and calcitonin gave negative results. Sections from 10% formaldehyde fixed materials, mounted and observed by fluorescent light, contained positive cells scattered in the urothelium (Fig. 2c). Such fluorescence was induced by the formaldehyde (FIF) cyclization of serotonin. Paraneurons are very numerous in the proximal third of the pelvic urethra, particularly in its pericollicular zone. Their number

Fig. 1. a. Human prostate. Grimelius, $\times 1300$. b. Rabbit prostate. Grimelius, $\times 1300$. c. Rabbit urethra. Masson-Hamperl, $\times 1600$. d. Buffalo prostate. Grimelius, $\times 1300$

decreases progressively in the middle and caudal thirds of the organ. Urethral neuroendocrine cells show wide diversity in their shape but the presence of various quanti-qualitative kinds of cytoplasmic processes seems to be a constant (Fig. 3a). In particu-

lar, serotonin-containing cells can have up to four dendrite-like projections, one of which is directed to the lumen. On the other hand, somatostatin-containing paraneurons show a unique process directed towards the basal membrane (Fig. 3b). Ovine

Fig. 2. Sheep urethra. a. Grimelius, $\times 450$. b. Linder, $\times 1600$. c. FIF, $\times 650$

prostatic paraneurons are much less numerous than the urethral ones. They are diffused throughout the gland, though more frequent in its proximal third, particularly in the pericollicular adenomeres and ducts. In the peripheral zones they are isolated or grouped in small clusters, as already described in the other species here examined.

The colocalization of 5-HT and chromogranin A in the same cells (Fig. 3c, d) was studied by means of the double labeling immunofluorescent technique applied on serial sections randomly collected from the

urethro-prostatic complex. Furthermore, serial sections were used to observe paraneurons along their entire extension and to establish more correctly the nature of their contents.

A first colocalization accounting of about two hundred paraneurons led us to conclude that almost half of such cells contain chromogranin A alone while one quarter contain serotonin alone and one quarter contain both substances colocalized.

Serotonin-, chromogranin A- and somatostatin-containing urethro-prostatic cells show the same

Fig. 3. Sheep urethra. **a.** Anti-5-HT (PAP-DAB) $\times 400$. **b.** Anti-somatostatin (PAP-AEC), $\times 1100$. **c.** and **d.** Double labeling: **c.** anti-chromogranin A (FITC), $\times 400$. **d.** anti-5-HT (LRITC), $\times 400$

distribution described for paraneurons in general. Somatostatin cells are much less numerous than the others in both the urethral and prostatic epithelia. Chromogranin A fluorescence was not enhanced by trypsinization.

As for as the female genital paraneurons, we shall report here detailed results from the uterus of the pig, and preliminary data from the uterus of the horse and mouse, a study still in progress. The results and data above are summarized in Table 3. The uterine horns of the pig were divided into thirds, proximal, middle and caudal, for the uniformity of sampling and description of results.

Argyrophil, non-argentaffin paraneurons were present in the uterine horns of all pigs examined. When immunohistochemical techniques were performed on sections of the same organ, immunoreactivities were found for chromogranin A and somatostatin (Fig. 4a, b) but not for 5-HT, VIP, bombesin, ACTH, gastrin and calcitonin. Immunopositive cells were never found in the uterine body.

In the normal pig, uterine paraneurons are contained in the adenomeres of the endometrial glands and in the initial portions of their excretory ducts. They are often grouped in small clusters and most numerous in the proximal third of the uterine horn, decreasing gradually in the middle and distal thirds. Their shape is generally round, though they sometimes show cytoplasmic processes interposed between the basal membrane and the exocrine epithelium.

Somatostatin immunoreactive cells are much less numerous than chromogranin A ones and therefore can be considered a subpopulation of the uterine paraneurons. The results described above were similar for impuberal and adult non-pregnant, and non-castrated pigs. The same results were observed in the uterine horn of hermaphroditic pig whose characteristics have already been described above.

In the proximal and middle thirds of the uterine horns of the horse (Fig. 4c) and mouse (Fig. 4d), argyrophil paraneurons were stained by means of the histochemical technique of Linder. Their distribution and cytomorphology are similar to those described for the same cells of the pig.

DISCUSSION

The serotonin-producing paraneuron is largely diffused in the male and female genital tract (FETISSOF et al., 1983, 1985; DI SANT'AGNESE et al., 1985; IWANAGA et al., 1987). Often such a cell is "open" in type, i.e., it shows a luminal projection by which "messenger molecules" in the lumen can contact the cell and induce a specific "answer" generally of a secretory type. On the basis of this recepto-secretory mechanism the paraneuron can be seen as the medium by which the organism can transform an external stimulus into an internal message causing functions of the body. Some authors maintain that an

Table 3. Paraneurons in the femal genital tract

Species-Organ			Argyrophil stains			Argentaffin stain	Antibodies			
			Grimelius	Linder	Bodian	Mass. Hamp.	Chrom. A	Somat.	Bomb.; ACTH	5-HT; VIP
Epithelio-chorial	Pig*-Uterus	horn	prox. 3rd	++	+++	+	—	++	+	—
			mid. 3rd	+	+	+	—	+	—	—
			dist. 3rd	+	+	—	—	+	—	—
	Horse-Uterus	horn	prox. 3rd	?	+	?	?	?	?	?
			mid. 3rd	?	+	?	?	?	?	?

*Normal and pseudohermaphroditic subjects.

Chrom. A: chromogranin A, Somat.: somatostatin, 5-HT:serotonin, VIP:vasoactive intestinal polypeptide, Bomb.: bombesin, ACTH:adrenocorticotrope hormone, Gastr.: gastrin, Calc.: calcitonin.

?: Not tested, —: not positive cells, +: rare positive cells, ++: few positive cells, +++: numerous positive cells.

open paraneuron producing serotonin can release the amine onto the surface of the lining epithelium and then in the organic lumen (GRONSTAD *et al.*, 1985; NILSSON *et al.*, 1987). Nevertheless some experimental data seem to confirm the first hypothesis; further research is needed to clarify this problem.

In various segments of the gastrointestinal tract the application of serotonin to the mucosal surface initiates peristalsis, while the application of the amine to the serosal surface inhibits it (GONELLA, 1981). Is it possible that the first is due to a paraneuronal serotonin effect and the second to a neuronal one? Of course, opposite actions of serotonin on smooth musculature can be due to the presence of different types of receptors (BRADLEY *et al.*, 1986).

The action of serotonin in the urogenital tract is not yet perfectly understood, but it is very probable that 5-HT plays a determinant role in the contraction/relaxation of various urogenital muscle structures. Such function is obviously important in the regulation of the release and flow of urogenital fluids, such as urine, sperm and surface secretions. On this basis the collicular zone of the male urethra, which contains numerous 5-HT-producing paraneurons in all mammalian species considered, is probably a segment having a determinant functional importance.

It has been reported that almost all the human urethro-prostatic paraneurons contain 5-HT and neuron specific enolase colocalized in the same cells (DI SANT'AGNESE *et al.*, 1985). 5-HT and chromogranin A

Fig. 4. **a** and **b.** Pig, uterine horn. Anti-somatostatin (PAP-DAB), $\times 1000$. **c.** Horse, uterine horn. Linder, $\times 1000$. **d.** Mouse, uterine horn. Linder, $\times 1000$

are colocalized also in the large majority of the analogous paraneurons of the dog (HANYU et al., 1987).

On the basis of our findings reported above, it is possible that the urogenital paraneurons of the ram might be a cellular population more polymorphous than those of the man and dog, with respect to their serotonin content and the distribution of chromogranins. The three chromogranins actually known, A, B and C, can be differently distributed in the same cytotype of various organs and animal species (RINDI et al., 1986).

In the normal urethral and prostatic epithelia of the man a small quantity of somatostatin- (DI SANT'AGNESE and DE MESY JENSEN, 1984), bombesin- (DI SANT'AGNESE, 1986), calcitonin- (DI SANT'AGNESE, 1986; FETISSOF et al., 1986a) and TSH- (ABRAHAMSSON et al., 1986) producing paraneurons have been described. The number of cells containing such peptides may be increased dramatically under pathological conditions such as carcinomas (CAPELLA et al., 1980; FETISSOF et al., 1986b; ABRAHAMSSON et al., 1987). This is the first description of a hormonal peptide, somatostatin, in urogenital paraneurons of non-human species. Such cells are rare and much less numerous than serotonin-producing ones. In particular, it has not been determined whether serotonin and somatostatin are colocalized in the same cells, as happens for serotonin and calcitonin in human genital paraneurons (FETISSOF et al., 1987).

The presence of paraneurons in the normal uterus was suspected when their presence was reported in the carcinogenic endometrium of humans (UEDA et al., 1980; FETISSOF et al., 1985). We have found paraneurons initially in the uterine horns of the pig and then in the same uterine segment of the horse and mouse. In such animal species the uterine horn must be considered as the site of pregnancy. The scope of our research program encompasses not only the study of the presence of uterine paraneurons, but also their correlation to placentation in different mammalian species, before and during pregnancy. On this basis the data collected in some earlier studies (VITTORIA et al., 1986b; PAINO et al., 1987; CECIO et al., 1988b; VITTORIA et al., 1989) and here, albeit partial, demonstrate the presence of paraneurons within the uterus of species showing both types of placentation considered: epitheliochorial (the pig and the horse) and hemochorial (the mouse).

The function of such cells in the uterus is unknown. It is possible that they regulate various functions in this organ which undergoes cyclic changes in its morphology. Such changes are extensive not only in species with hemochorial placentae but also in those

with epitheliochorial ones, as the pig (STROBAND et al., 1986).

Actually, it is clear that present in the "matrix" is a complex of factors, largely unknown, which play a role in embryological and foetal nutrition and development. One or some such factors are for example contained in the uterine exocrine secretion of the pig (SINOWATZ and FRIESS, 1983; STROBAND et al., 1986). For this reason, uterine paraneurons could also have important functions during pregnancy.

The embryonal origin of the urogenital paraneurons was considered endodermal because they had only been found in organs derived from the urogenital sinus (DI SANT'AGNESE and DE MESY JENSEN, 1984; HANYU et al., 1987). Our finding of such cells in the uterus allows us to view the urogenital paraneurons as also mesodermal in origin.

Somatostatin-containing cells are described here both in the male and female urogenital tracts. The functional role of these cells in such organs is unknown. It is certain that in the gastroenteropancreatic system this hormone is released through cytoplasmic processes in the intercellular spaces where it inhibits the endocrine and exocrine secretion of nearby cells (LARSSON et al., 1979; REICHLIN, 1983a, b). This modality of action is notoriously called "paracrine". The somatostatin-containing cells of the porcine uterus are surrounded by endocrine and exocrine cells and often show dendrite-like cytoplasmic processes. They probably have a paracrine function. On the other hand, the somatostatin immunoreactive cells of the ovine urethra have a traditional endocrine function: in fact, their processes are constantly directed towards the capillaries of the subepithelial connective tissue.

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